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Reduced Glycosylation of Human Cell Lines Increases Susceptibility to CD4-Independent Infection by Human Immunodeficiency Virus Type 2 (LAV-2/B)

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The human immunodeficiency virus type 2 (HIV-2) strain LAV-2/B is able to infect a variety of human cell lines via a CD4-independent pathway. We have used the glycosylation inhibitors tunicamycin, swainsonine, and deoxymannojirimycin to further characterize this putative alternative receptor for HIV-2 (LAV-2/B). These antibiotics resulted in an increase (5- to 30-fold) in the susceptibility of a variety of CD4[−] human cell lines to infection by LAV-2/B (RD, HeLa, HT29, Rsb, Heb7a, Hos, and Daudi). Several nonprimate cell lines (mink Mv-1-lu, rabbit SIRC, hamster a23, mouse NIH 3T3, cat CCC, and rat HSN) remained resistant to infection by LAV-2/B after treatment with glycosylation inhibitors, suggesting that they do not express the HIV-2 CD4-independent receptor. Two of these nonprimate cell lines are readily infected by HIV-2 when they express CD4 (Mv-1-lu and CCC). Treatment of human cells with neuraminidase had no effect on subsequent infection by LAV-2/B, suggesting that the increase in susceptibility to infection of deglycosylated cells is not due to a change in the electrostatic charge of the cell surface. Treatment of RD CD4[−] cells and HeLa CD4⁺ cells with a variety of proteases resulted in a 75 to 90% decrease in infection by LAV-2/B when compared with untreated cells. Taken together, all these data suggest that HIV-2 can utilize a membrane glycoprotein other than CD4 to attach and fuse with a variety of human cells.

The CD4 molecule is the principal cellular receptor for the human and simian immunodeficiency viruses (HIV-1, HIV-2, and SIV) (15, 24, 40). However, attachment of HIV or SIV to CD4 on the cell membrane is not always sufficient for infection. Several examples exist in which HIV or SIV binding to CD4⁺ human or nonhuman cells does not lead to membrane fusion or infection (1, 2, 6, 8, 28, 29). The implications of these experiments are that cell surface components other than CD4 are necessary for HIV entry. Furthermore, many cell lines of nervous system, liver, and fibroblast origin do not express CD4 but can be infected, albeit less efficiently, with one or more strains of HIV-1 (7, 10, 16, 19, 20, 45). It has been reported that a selected strain of HIV-2 (LAV-2/B) can efficiently direct cell-to-cell fusion and infection of certain CD4-negative human cell lines (9). Infection of CD4-negative cells by other HIV-2 strains was induced or greatly enhanced by the presence of soluble CD4 (sCD4). The CD4 molecule therefore appears to induce conformational changes in the virus envelope glycoproteins (41) which may be necessary for fusion of viral and cellular membranes.

Here we report on the biochemical characterization of the receptor involved in HIV-2 CD4-independent entry into cells. These data show that inhibition of complex glycan synthesis on membrane glycoproteins increases the rate of infection of certain human cells by HIV-2 via the CD4-independent receptor. Steric blocking of virus-binding sites on the receptor by complex glycans and different glycosylation patterns among human cell lines may account for the selective tropism pattern of LAV-2/B on CD4-negative cells. Furthermore, the protease sensitivity of CD4-negative cells points to the presence of a

membrane protein in the CD4-independent entry pathway of HIV-2.

MATERIALS AND METHODS

Cells. The human T-cell line, H9 (38), and B-cell line, Daudi (37), were both maintained as suspension cultures in RPMI 1640 medium (Gibco), supplemented with 10% (vol/vol) fetal calf serum (FCS), 60 µg of penicillin per ml, and 100 µg of streptomycin per ml. The human rhabdomyosarcoma cell line RD (TE671 subline) (43), the human carcinoma lines HeLa, Heb7a, HT-29, Rsb, and SCL1, the osteosarcoma cell line HOS (6, 8, 9), the fibrosarcoma cell line HT1080 (39), the human fibroblast cell line WI38 (6) and nonprimate fibroblast cell lines (i) rabbit corneal cell line SIRC (27), (ii) Chinese hamster lung fibroblastoid cell line a23 (46), (iii) mouse cell line NIH 3T3 (29, 30), (iv) cat kidney cell line CCC (12), (v) rat sarcoma cell line HSN (13), and (vi) mink lung cell line Mv-1-lu (23) were all maintained as monolayers in Dulbecco's modified Eagle's medium (Gibco) supplemented with 4% (vol/vol) FCS, 60 µg of penicillin per ml, and 100 µg of streptomycin per ml. HeLa CD4⁺ (28) cells were maintained in the same medium supplemented with 1 mg of geneticin (Gibco) per ml.

Viruses. The HIV-2 strain LAV-2/B (9) was derived from C8166 cells chronically infected with LAV-2_{ROD} (11). The HIV-2 strain CBL-22 has been described previously (9).

Antibodies. The monoclonal antibody to the V1 domain of human CD4 (22) was supplied by the Medical Research Council AIDS Reagent Programme (repository reference ADP318). The neutralizing monoclonal antibody to LAV-2/B SU glycoprotein, 8e, and the pooled rat monoclonal antibodies against LAV-2/B envelope used for immunostaining were gifts from Aine McKnight (29a).

Treatment of cells with glycosylation inhibitors. Cells were seeded at 10⁵ cells per ml on 24-well trays and grown for 24 h at 37°C; then, the medium was replaced with medium containing 0.1 µg of tunicamycin per ml, 0.1 mM swainsonine, or 1 mM 1-deoxymannojirimycin (Sigma). The cultures were allowed to grow for a further 24 h, and then the medium was removed and fresh medium containing serially diluted virus was added. The virus was allowed to adsorb for 5 h at 37°C, when it was replaced with more fresh medium. After overnight incubation, 1 µg of azidothymidine (AZT; Sigma) per ml was added; the infected cells were immunostained after a further 3 days of incubation.

Treatment of virus with monoclonal antibodies and sCD4. LAV-2/B was neutralized with the rat MAb 8e (final concentration, 1 µg/ml) for 1 h at 37°C prior to addition to cells. In experiments with sCD4 (kindly provided by Ray Sweet, SmithKline Beecham), virus was incubated for 1 h with 10 µg of sCD4 per ml at 37°C prior to addition to cells. Cells were treated with anti-CD4 monoclonal antibody (5 µg/ml) for 24 h prior to infection with virus.

Neuraminidase treatment of cells. Cells were seeded at 10⁵ cells per ml on 24-well plates and grown for 24 h at 37°C. The cells were washed twice in

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complete phosphate-buffered saline (cPBS)–0.05% FCS, and then 200 μ l of bacterial neuraminidase solution (0.2 U/ml in cPBS–0.05% FCS) from either *Salmonella typhimurium* (Sigma) or *Vibrio cholerae* (Boehringer Mannheim) or cPBS–0.05% FCS alone was added to the wells. After incubation at 37°C for 1 h, the cells were washed three times with cPBS. LAV-2/B was titrated on the cells and allowed to adsorb for 2 h at 4°C (incubation at this temperature was used to allow virus attachment but prevent cells from regenerating glycoproteins containing terminal sialic acid residues). After overnight incubation, AZT (1 μ g/ml) was added; the infected cells were immunostained after a further 3 days of incubation.

An agglutination assay was used to assess the cleavage of terminal sialic acid residues by the bacterial neuraminidase. Approximately 10^6 cells were treated with neuraminidase (0.2 U/ml in cPBS–0.05% FCS) and then aliquoted onto round-bottom 96-well plates (10^5 cells per well). Lectin from *Limulus polyphemus* (Sigma) was titrated (fourfold serial dilutions in cPBS–100 mM CaCl₂) onto the cells, and the plates were incubated for 30 min at 37°C.

Protease treatment of cells. Cells were removed with Versene (0.02% [wt/vol] in PBS), washed once with cPBS–0.1% (wt/vol) EDTA, and incubated with trypsin (1 mg/ml; Sigma), α -chymotrypsin (0.02 mg/ml; Sigma), or bromelain (0.02 mg/ml; Sigma) diluted in cPBS–0.1% (wt/vol) EDTA (2×10^6 cells per ml) for 10 min at 37°C. Soybean trypsin inhibitor (type II-S; Sigma), chymotrypsin-trypsin inhibitor (Sigma), or bromelain inhibitor (Sigma) was added at 1 mg/ml, and the cells were washed three times with cPBS–0.1% (wt/vol) EDTA at 4°C. The cells were finally resuspended in complete medium and plated onto 24-well trays (200 μ l per well; 4×10^5 cells). LAV-2/B was titrated onto the cells and allowed to adsorb at 4°C for 2 h (incubation at this temperature was used to keep regeneration of cleaved surface proteins to a minimum). At the end of this period, medium containing unbound virus was removed and added to untreated cells to determine virus viability. After overnight incubation, AZT (1 μ g/ml) was added; the infected cells were immunostained after a further 3 days of incubation.

Cell-free virus infections. Virus supernatant stocks were serially diluted in fivefold steps and added in 250- μ l amounts to appropriate cell types in 24-well trays. Unless otherwise stated, the virus was allowed to adsorb for 5 h at 37°C before unbound virus was removed and replaced with appropriate medium. After overnight incubation, AZT (1 μ g/ml) was added; the infected cells were immunostained after a further 3 to 5 days of incubation.

Immunostaining HIV-2-infected cells. Infection assays were typically performed in 24-well tissue culture trays. Three days after infection, the medium was removed and the cells were washed twice in PBS before being fixed with a 50:50 mixture of methanol-acetone at –40°C for 2 min. After removal of the methanol-acetone, the cells were washed once with PBS followed by PBS containing 1% (vol/vol) FCS. The primary antibody (pooled rat monoclonal antibodies against LAV-2/B envelope; 250 μ l per well, diluted 1:50 in PBS–1% FCS) was added to the fixed cells, allowed to bind for 1 h at room temperature, and then washed twice in PBS–1% FCS. Goat anti-rat immunoglobulin G– β -galactosidase conjugate (Sera-labs) at a 1:600 dilution (in PBS–1% FCS) was added for 1 h at room temperature, and the mixture was washed twice in serum-free PBS. Approximately 500 μ l of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) substrate (0.5 mg/ml) in PBS containing 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 1 mM magnesium chloride was added to each well. Infected cells stained blue within 1 to 2 h of the addition of substrate.

RESULTS

Throughout this study, an immunostaining procedure (9) has been used to assess the infection and replication of HIV-2 in a variety of CD4-negative cells pretreated with glycosylation inhibitors, protease, or bacterial neuraminidase. The secondary antibody in this assay is conjugated to β -galactosidase, which stains infected cells blue upon addition of the substrate (X-Gal). The immunostaining procedure proved very specific for HIV-2-infected cells, with no background staining or staining of uninfected cells being observed (Fig. 1). In all experiments, virus was titrated onto the cells by fivefold serial dilution steps, and the apparent titers were determined by counting the number of foci of infection. To prevent the possibility of secondary infections increasing the observed virus titer, the reverse transcriptase inhibitor AZT was included in the incubation medium. Titers have been expressed as focus-forming units per milliliter.

Figure 1 shows the immunostaining of a number of LAV-2/B-infected human cells. Infection of RD cells and HeLa CD4⁺ cells results in significant cell-to-cell fusion with formation of large syncytia. LAV-2/B infection of HeLa and HOS cells produced a majority of singly infected cells and a very few

small syncytia, whereas infection of HT-29, Rsb, and Heb7a cells produced only singly infected cells and no cell-to-cell fusion (Table 1).

Treatment of a variety of human cells with glycosylation inhibitors increases the susceptibility to infection by HIV-2 via the CD4-independent pathway. We have tested the infectibility of a panel of CD4-negative human cells by HIV-2 after treatment with the glycosylation inhibitors tunicamycin, swainsonine, and 1-deoxymannojirimycin. Tunicamycin inhibits the first reaction in the lipid-saccharide pathway (synthesis of *N*-acetylglucosaminyl pyrophosphoryl polyisoprenol), blocking *N*-linked glycosylation completely (44). The antibiotics 1-deoxymannojirimycin and swainsonine inhibit the mannose-trimming enzymes mannosidase IA/B and mannosidase II, respectively, and therefore prevent the subsequent synthesis of complex glycan structures (18). Tunicamycin was observed to inhibit cell growth in comparison with untreated cells, whereas swainsonine and 1-deoxymannojirimycin had little effect. Tunicamycin treatment of certain CD4-negative human cells (RD, HeLa, HOS, Rsb, HT-29, and Heb7a) increased the apparent titer of LAV-2/B by between 5- and 30-fold (Fig. 2). Conversely, tunicamycin pretreatment of RD or HeLa cells did not remove the block to infection by HIV-1 (IIIb) (results not shown). Pretreatment of human cells with the antibiotics swainsonine and 1-deoxymannojirimycin resulted in a smaller increase in the apparent titer of LAV-2/B of up to fivefold (Fig. 2). However, none of the three antibiotics used had any effect on the infectability of HeLa CD4⁺ cells by LAV-2/B, although infection by HIV-1 (IIIb) was decreased approximately fourfold after pretreatment of the cells with tunicamycin. This observation is consistent with down regulation of the surface expression of CD4 as a result of selective degradation of partially glycosylated molecules after tunicamycin treatment (26). The effective increase in infection of tunicamycin-treated HeLa cells by LAV-2/B via the CD4-independent receptor (and presumably HeLa CD4⁺ cells) apparently cancels the effect of the down regulation of CD4.

The CD4-independent infection of RD cells by strain CBL-22 is increased 30-fold after treatment of the cells with tunicamycin. Clapham et al. (9) have shown that another strain of HIV-2 (CBL-22) can fuse RD cells in a CD4-independent manner. We have determined the titer of CBL-22 on a variety of human cells treated with tunicamycin. The results in Table 2 show that tunicamycin pretreatment of RD cells increases the titer of CBL-22 by 30-fold. This is in contrast to the low rate of infection of HeLa, Rsb, and Heb7a cells by CBL-22, which was not enhanced after treatment with tunicamycin. CBL-22 showed no infection of five other human cell lines (HT29, HOS, SCL1, HT1080, and WI38).

sCD4 further enhances the susceptibility of tunicamycin-treated CD4-negative cells to infection by HIV-2. It has been reported previously that pretreatment of several HIV-2 strains with sCD4 enhances the fusion of CD4-negative RD (TE671) cells (9). Figure 3 shows that infection of tunicamycin-treated RD cells is further enhanced in the presence of sCD4 (10 μ g/ml). This is most dramatically indicated when comparing the infection of untreated RD cells by LAV-2_{ROD} with the infection of tunicamycin-treated RD cells by LAV-2_{ROD} in the presence of sCD4 (50-fold enhancement). Conversely, infection of HeLa CD4⁺ cells by both LAV-2/B and LAV-2_{ROD} is inhibited in the presence of sCD4. This inhibitory effect is partially alleviated when the cells are pretreated with tunicamycin, presumably because this increases the availability of the CD4-independent receptor as a route for infection.

The increased sensitivity to infection by HIV-2 is a receptor-specific process. To test the possibility that tunicamycin acts in

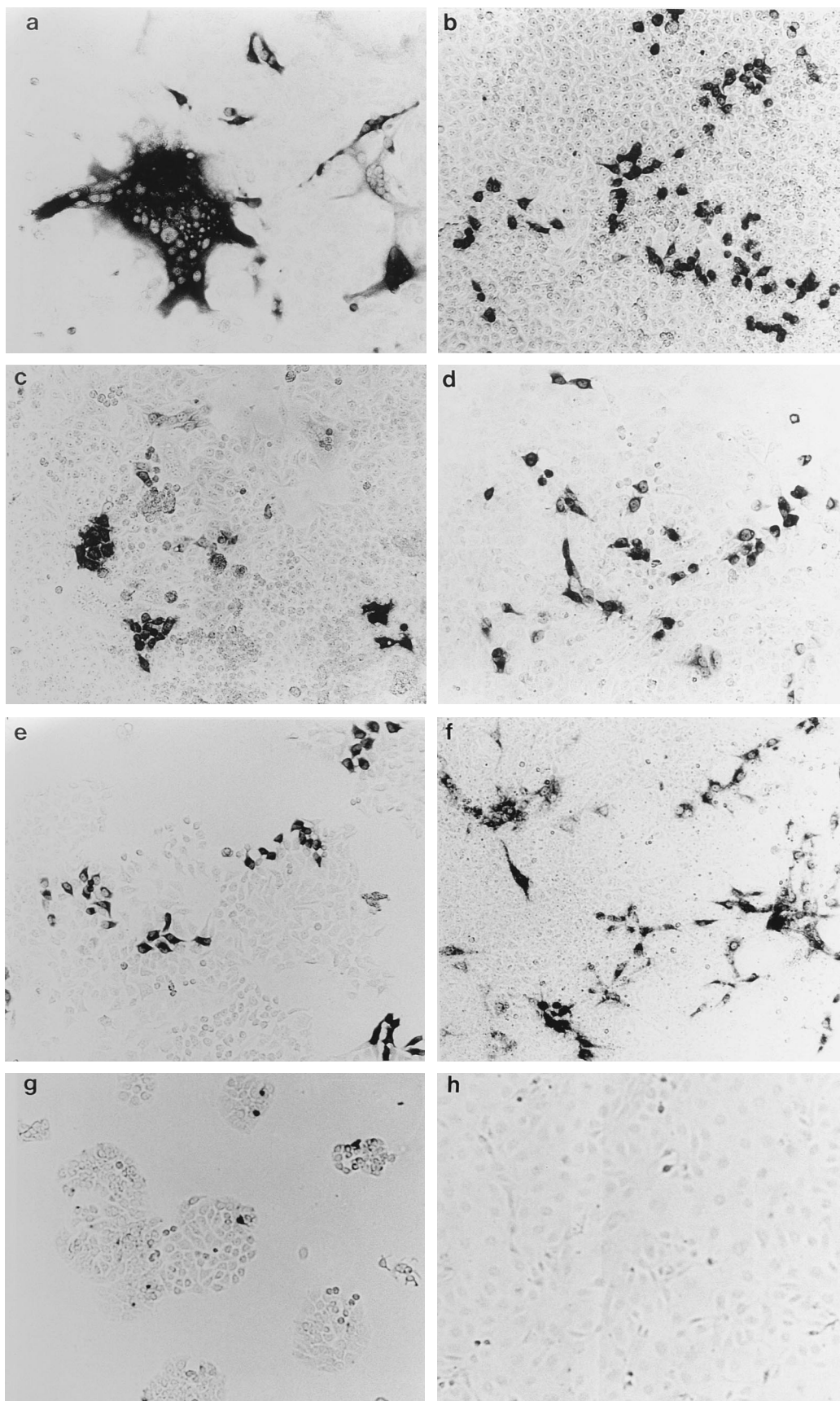


FIG. 1. Immunostaining of CD4-negative human cells after infection with LAV-2/B. Cells were stained as described in Materials and Methods, with infected cells appearing black in the images. (a) RD (TE671); (b) HeLa; (c) HeLa CD4⁺; (d) HOS; (e) Heb7a; (f) Rsb; (g) HT-29; (h) SCL1. The human cells SCL1 (panel h), WI38, and HT1080 and the nonprimate cells SIRC, CCC, HSN, Mv-1-lu, NIH 3T3, and a23 were all uninfected with LAV-2/B. Pretreatment of RD or HeLa cells with tunicamycin did not allow infection by HIV-1 (IIIb).

TABLE 1. Infection and syncytium induction on tunicamycin-pretreated human cells by LAV-2/B

Human cell line	Titer (FFU/ml)	% of infected cells forming syncytia
HeLa CD4 ⁺	7.5×10^3	50–75
RD	3.3×10^4	>90
HeLa	2.8×10^4	≤1
HOS	2.4×10^3	≤1
Rsb	1.6×10^3	0
Heb7a	3.6×10^4	0
HT-29	1.7×10^3	0
SCL1	0	0
WI38	0	0
HT1080	0	0
Six nonprimate cell lines ^a	0	0

^a Mv-1-lu, CCC, HSN, a23, SIRC, and NIH 3T3.

some nonspecific manner to promote infection of CD4-negative cells by HIV-2, we attempted to infect various tunicamycin-treated nonprimate cells with LAV-2/B. None of the nonprimate cells were infected by LAV-2/B after treatment with tunicamycin (Table 1), although some will allow LAV-2/B infection and replication when human CD4 is expressed on the cell surface (CCC and Mv-1-lu [29]). These data indicate that the HIV-2 CD4-independent receptor is not expressed on the nonprimate cells tested (and also certain human cells, e.g., HT1080, WI38, and SCL1 [Table 1]) in a form recognizable by

TABLE 2. Comparison of the titer of CBL-22 (HIV-2) on untreated human cells and tunicamycin-treated human cells

Cell line	Titer of CBL-22 (FFU/ml) on:	
	Untreated cells	Treated cells
RD	1.6×10^3	5.2×10^4
HeLa CD4	3.4×10^3	4.6×10^3
HeLa	2.0×10^2	1.6×10^2
Heb7a	4.0×10^1	6.0×10^1
Rsb	4.0×10^1	4.0×10^1
HOS, HT29, SCL1, WI38, and HT1080	0	0

LAV-2/B and that the effect of tunicamycin on other human cells is specific. Furthermore, LAV-2/B infection of tunicamycin-treated CD4-negative cells is completely blocked by the neutralizing monoclonal antibody 8e (Fig. 4). This monoclonal antibody specifically blocks entry of LAV-2/B into cells via the CD4-independent pathway (29a). Figure 4 shows that 8e does not block entry of LAV-2/B into HeLa CD4⁺ cells except when they have been pretreated with tunicamycin. This may be explained by the observation that tunicamycin can lead to down regulation of the expression of CD4 on the cell membrane, as a result of a selective degradation of unglycosylated CD4 molecules (26). A monoclonal antibody against human CD4 does not block infection of CD4-negative cells, whether or not they have been pretreated with tunicamycin. However, anti-CD4 does block infection of untreated HeLa CD4⁺ cells. Pretreatment of HeLa CD4⁺ cells with tunicamycin removes the subsequent block to infection by anti-CD4, since LAV-2/B is able to utilize the CD4-independent receptor to gain entry.

The low rate of infection of many human cell types is not due to any secreted inhibitory factors. It has been reported that CHO-K1 cells (31) secrete a soluble factor (possibly an endogenous retroviral Env protein) which is responsible for blocking infection by murine leukemia virus and amphotrophic pseudotypes of gibbon ape leukemia virus. We have investigated the possibility that the low level of HIV-2 infection of some human cells is due to a secreted soluble inhibitory factor rather than to the heterogeneity of glycosylation of the receptor on different cell types. Serial dilutions of LAV-2/B were incubated for 1 h in either HeLa cell conditioned medium or untreated medium and then added to RD cells on 24-well trays which had also been incubated for 1 h with HeLa cell conditioned medium or untreated medium. The apparent titer of LAV-2/B was not altered by preincubation of virus or cells in HeLa cell conditioned medium, suggesting that HeLa cells do not secrete any factors which interfere with HIV-2 infection.

Removal of terminal sialic acid residues from cell surface glycoproteins has no effect on infection by HIV-2. Sialic acid on the surface of RD, HeLa, and HeLa CD4⁺ cells is not required for the attachment of LAV-2/B, since cleavage of terminal sialic acids by neuraminidase from *V. cholerae* or *S. typhimurium* had no significant effect on infection (Fig. 5a). Neuraminidases from *V. cholerae* and *S. typhimurium* have preferences for cleaving α-2,6- and α-2,3-linked sialic acid residues, respectively (21).

The effectiveness of the neuraminidase enzymes in cleaving terminal sialic acid residues was assessed in parallel with an agglutination assay involving a lectin from *L. polyphemus* (which binds sialic acid residues specifically). The lectin agglutinated untreated RD, HeLa, and HeLa CD4⁺ cells at a concentration of 10 to 20 μg/ml, compared with >100 μg/ml after treatment of the cells with bacterial neuraminidase. This indi-

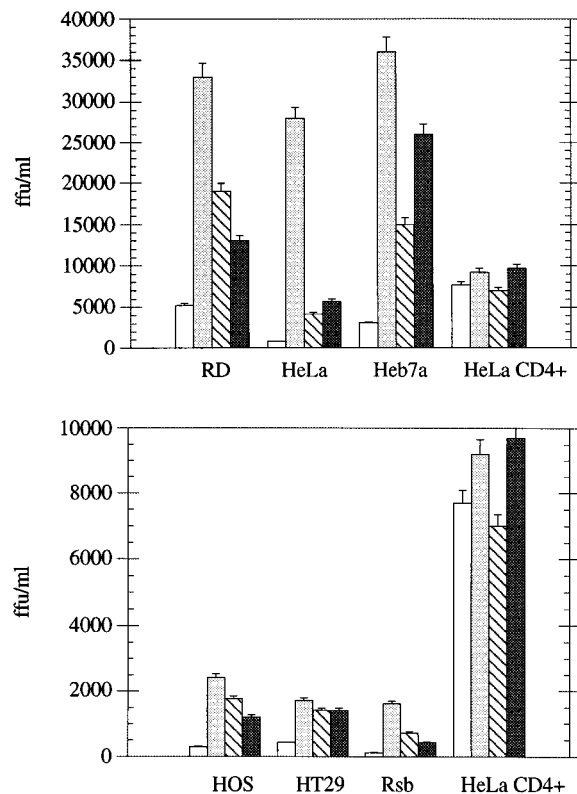


FIG. 2. LAV-2/B cell-free virus titer determinations on human CD4-negative and HeLa CD4⁺ cell lines pretreated with glycosylation inhibitors. The titers (focus-forming units [ffu] per milliliter) are arithmetic means and standard deviations for triplicate parallel samples on cells treated with 0.1 μg of tunicamycin per ml (▨), 0.1 mM swainsonine (▤), or 1 mM 1-deoxymannojirimycin (■) or untreated (□).

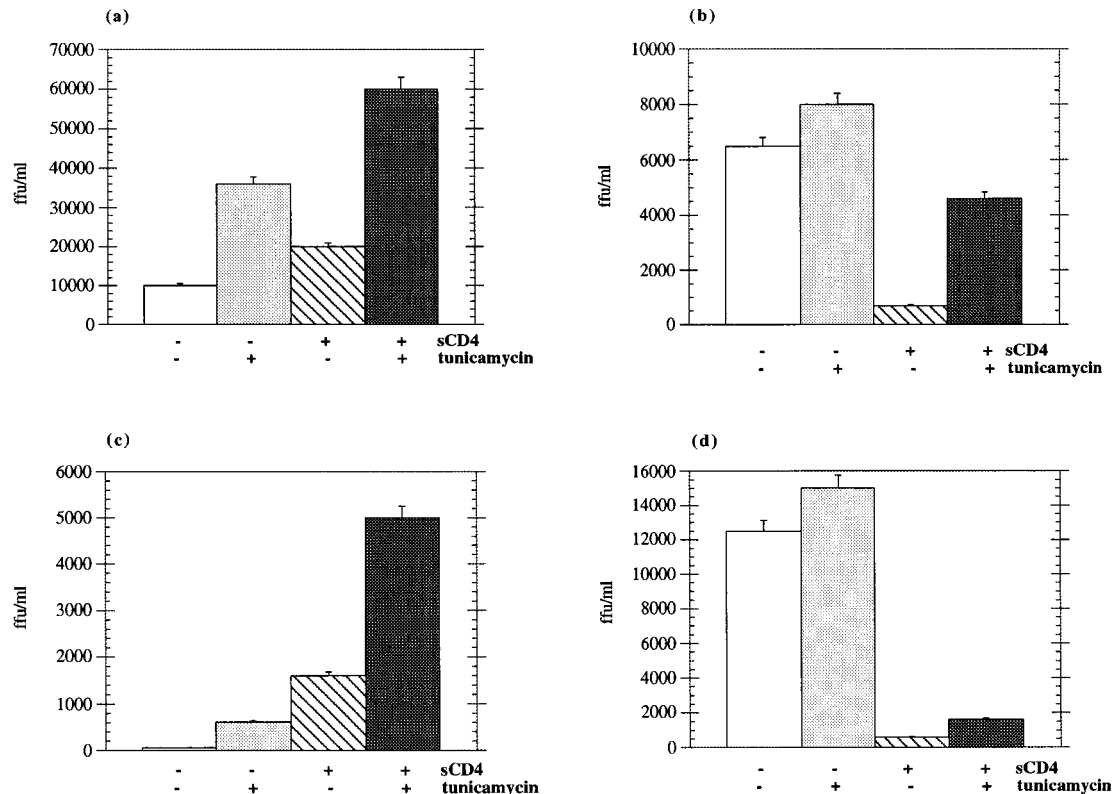


FIG. 3. sCD4 sensitivity of HIV-2 cell-free virus titer determinations on RD (TE671) and HeLa CD4⁺ cells. Cells were pretreated with tunicamycin (0.1 μ g/ml) for 24 h. HIV-2 was preincubated with sCD4 (10 μ g/ml) for 1 h prior to infection of cells. (a) LAV-2/B infection of RD (TE671) cells. (b) LAV-2/B infection of HeLa CD4⁺ cells. (c) LAV-2_{ROD} infection of RD (TE671) cells. (d) LAV-2_{ROD} infection of HeLa CD4⁺ cells. Titers (focus-forming units [ffu] per milliliter) are expressed as arithmetic means and standard deviations for triplicate samples.

cates that a significant proportion of the terminal sialic acid residues have been cleaved under the conditions used.

Treatment of cells with protease decreases the apparent titer of LAV-2/B by up to 10-fold. The biochemical nature of the LAV-2/B-binding moiety on CD4-negative cells was fur-

ther characterized by incubation of RD cells with several proteases (Fig. 5b). Following proteolytic cleavage of membrane proteins, the proteases were inactivated with an excess of inhibitor and the cells were washed extensively. Trypsin-, α -chymotrypsin-, and bromelain-treated cells were infected 65, 80, and 90% less, respectively, than control cells were. This indicates that a cell surface protein is an essential component of the CD4-independent HIV-2 binding or fusion process. Protease-treated HeLa CD4⁺ cells showed a similar reduction (70 to 92%) in infection by LAV-2/B (results not shown).

The viability of the virus after incubation with protease-treated cells was tested by replating the virus on pretreated cells. The titer of residual LAV-2/B remained the same (Fig. 5b, panel B) after incubation with either protease-treated or mock-treated cells, indicating that all traces of protease had been removed by the use of inhibitors and extensive washes.

The viability of cells after protease treatment was addressed by digesting cell surface proteins after virus entry. The titer of LAV-2/B on RD cells was identical after protease treatment or mock treatment of the cells 5 h after addition of virus (results not shown).

DISCUSSION

It was reported previously that HIV-2 LAV-2/B can infect RD cells via a CD4-independent pathway (9). We have attempted to characterize the cell surface receptor responsible by several biochemical methods. Treatment of a variety of human cells (Fig. 2) with the glycosylation inhibitor tunicam-

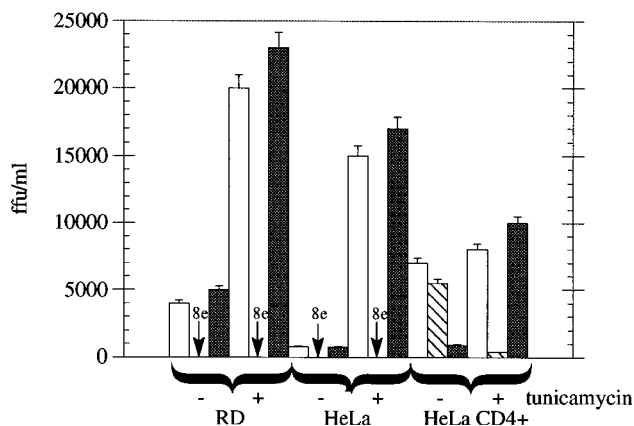


FIG. 4. Antibody sensitivity of LAV-2/B cell-free virus titer determinations on RD, HeLa, or HeLa CD4⁺ cells. Cells were pretreated with tunicamycin (0.1 μ g/ml) and/or anti-CD4 (5 μ g/ml) for 24 h prior to virus titer determinations. LAV-2/B was neutralized for 1 h with anti-LAV-2/B (8e) prior to titer determinations on cells. Titers (focus-forming units [ffu] per milliliter) are expressed as arithmetic means and standard deviations for triplicate samples. Symbols: \square , no antibody; \square with 8e, anti-LAV-2/B 8e; \blacksquare , anti-CD4.

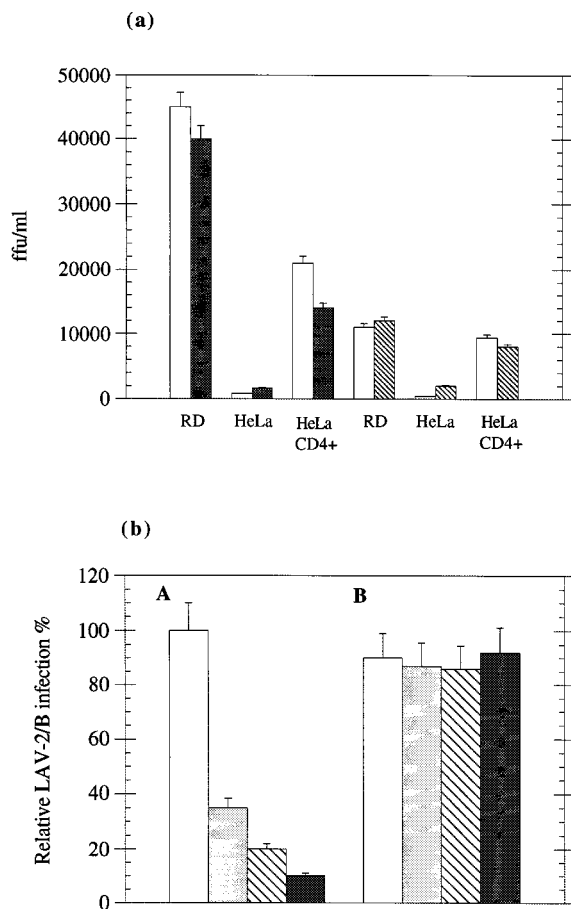


FIG. 5. Neuraminidase and protease sensitivity of LAV-2/B infection of RD, HeLa, and HeLa CD4⁺ cells. (a) Cells were pretreated with neuraminidase from *S. typhimurium* (■) or *V. cholerae* (▨) or cPBS-0.05% FCS alone (□), washed, and subsequently infected with LAV-2/B for 2 h at 4°C. Titers (focus-forming units [ffu] per milliliter) are expressed as arithmetic means and standard deviations for triplicate samples. (b) In panel A, RD cells were incubated for 10 min at 37°C in cPBS-0.1% EDTA alone (□) or with trypsin (1 mg/ml [▨]), α-chymotrypsin (0.02 mg/ml [▨]), or bromelain (0.02 mg/ml [■]), all diluted in cPBS-0.1% EDTA, washed, and subsequently infected with LAV-2/B for 2 h at 4°C. In panel B, the relative infection of RD cells by residual unbound virus remaining after incubation with untreated and protease-treated cells (cPBS/0.1% EDTA alone [□]; trypsin [▨]; α-chymotrypsin [▨]; bromelain [■]) is shown. Values in both panels represent arithmetic means and standard deviations for triplicate samples, expressed as percentages of values obtained from infected control cells.

cin, swainsonine, or 1-deoxymannojirimycin results in a significant increase in the apparent titer of LAV-2/B. This increase in infectibility appears to be specific to the HIV-2 interaction with the CD4-independent receptor, since tunicamycin does not promote infection of some other human (WI38, SCL1, and HT1080) and nonprimate (NIH 3T3, HSN, CCC, Mv-1-lu, a23, and SIRC) cells (Table 1). This is most probably due to the nonexpression of the CD4-independent receptor on these cell types, since HIV-2 is able to infect and replicate in some of these cells when they express human CD4 on the cell surface (6, 29). The infection of RD cells by a different HIV-2 strain (CBL-22) was enhanced 30-fold after treatment of the cells with tunicamycin (Table 2). This demonstrates that although infection of cells by the CD4-independent route is most efficient with LAV-2/B, other strains of HIV-2 are also capable of infecting CD4 negative cells. Several other cell lines (Table 2), however, had a very low rate of infection or were uninfected by CBL-22, even after treatment with tunicamycin.

The removal of the block to infection of certain human cell lines by inhibitors of complex glycan synthesis may go some way toward explaining the observed cell tropism pattern of LAV-2/B. The endogenous receptors for LAV-2/B on these cell types are nonfunctional but become functional after treatment of the cells with glycosylation inhibitors. One possibility is that these receptors are rendered nonfunctional by addition of sugar residues during synthesis that block virus binding or internalization. The LAV-2/B envelope must interact with the polypeptide backbone of the receptor, with the site of interaction becoming more or less blocked by complex glycan structures depending on the variation in glycosylation patterns in different cells. The putative interaction with the polypeptide rather than the sugar moiety of the receptor is emphasized by the varying increase in infection of human cells when treated with tunicamycin compared with 1-deoxymannojirimycin and swainsonine. Treatment of cells with tunicamycin blocks all N-linked glycosylation, since it inhibits the synthesis of the high-mannose lipid-linked core glycan to which complex carbohydrate structures are added subsequently (44). This would leave the polypeptide backbone fully exposed for interaction with the LAV-2/B envelope. Swainsonine and 1-deoxymannojirimycin, on the other hand, inhibit mannose-trimming enzymes (mannosidase II and IA/B respectively) and therefore block the conversion of high-mannose to complex oligosaccharide-type structures (18). This would result in glycoproteins with truncated high-mannose carbohydrates capable of partially blocking the interaction of LAV-2/B envelope with the receptor; this explains the lower increase in the apparent titer of LAV-2/B on cells treated with these two antibiotics.

It has been reported previously that tunicamycin treatment of CEM cells leads to a down regulation of surface expression of CD4 molecules (26). This was shown to be due to the preferential intracellular degradation of the unglycosylated precursor. In the same study, König et al. (26) did not observe any inhibition of surface expression of three other membrane glycoproteins (namely, CD2, CD5, and the human transferrin receptor) after treatment of cells with tunicamycin. Other groups have noted that tunicamycin prevents some but not all glycoproteins from being secreted or reaching the cell surface (32, 34, 35, 42). Another possible explanation, therefore, for the increase in the titer of LAV-2/B on CD4-negative cells treated with tunicamycin is that we are observing a down regulation in the membrane expression of a glycoprotein which normally inhibits HIV-2 entry into CD4-negative cells. We have ruled out the possibility of a soluble factor which acts to inhibit LAV-2/B infection by pretreating cells or virus with HeLa cell conditioned medium. The presence of a membrane-bound inhibitory protein would also seem unlikely, since both swainsonine and 1-deoxymannojirimycin also increase the infectibility of CD4-negative cells by LAV-2/B; neither of these antibiotics has been shown to significantly affect the surface expression of glycoproteins (18).

HIV-2 infection of tunicamycin-treated RD cells was further enhanced in the presence of sCD4 (Fig. 3). This was more dramatically illustrated with HIV-2 LAV-2_{ROD} than with LAV-2/B, since LAV-2_{ROD} normally shows only a limited infection of RD cells. Previous studies have shown that sCD4 enhances HIV-2 and SIV infection of CD4-negative cells (9). Binding of sCD4 to HIV-1 gp120 results in conformational changes which alter the exposure of the V3 loop to binding of V3 antibodies (41). We could speculate that the enhancement of HIV-2 infection of CD4-negative cells is due to the interaction of sCD4 with a homologous V3 region, resulting in the exposure of a buried site on gp110 for receptor binding. It is therefore probable that this receptor-binding site is already

exposed on LAV-2/B envelope protein, since significant infection of CD4-negative cells is observed in the absence of sCD4. The observation that sCD4 inhibits the entry of HIV-2 into HeLa CD4 cells provides further evidence that the HIV-2 envelope interacts with the CD4-independent receptor via a site distinct from the site which binds to CD4.

Treatment of RD cells with two different bacterial neuraminidases with preferences for different terminal sialic acid linkages (neuraminidase from *V. cholerae* preferentially cleaves α -2,6-linked sialic acid residues, and neuraminidase from *S. typhimurium* preferentially cleaves α -2,3-linked sialic acid residues) had no significant effect on subsequent infection of RD, HeLa, or HeLa CD4⁺ cells by LAV-2/B. It has been shown that terminal sialic acid residues are required for the attachment of members of the polyomavirus genus to host cells, with a preference for either α -2,6- or α -2,3-linked sialic acid residues (4, 17, 21). The fact that removal of terminal sialic acid residues from membrane glycoproteins had no effect on binding and infection of LAV-2/B suggests that charge repulsion does not play a role in preventing entry by LAV-2/B into some CD4-negative cells.

The protease sensitivity of HIV-2 attachment and/or entry into CD4-negative cells indicates the involvement of a membrane protein in the binding or fusion event. Furthermore, the enhancement of infection when cells are treated with glycosylation inhibitors suggests that this membrane protein is usually glycosylated to different degrees, depending on the cell type. Harouse et al. (19) have identified a galactocerebroside on CD4-negative neural cells to which gp120 of HIV-1 binds specifically and with high affinity, suggesting a role in HIV entry into neural cells. Two HIV-1 gp120-binding proteins have been identified by Berberian et al. (3) (immunoglobulin V_H3 gene products) and Curtis et al. (14) (membrane-associated C-type lectin), although neither of these proteins promoted HIV internalization into cells. It is therefore debatable whether these proteins play any significant role in CD4-independent infection of cells by LAV-2/B. The putative CD4-independent receptor for HIV-2 described here appears to consist of at least one membrane-associated glycoprotein, although we cannot rule out the possibility of other membrane molecules playing an additional role in either binding or fusion.

It is not clear whether the CD4-independent infection of certain human cells by HIV-2 has significant in vivo implications. There is limited evidence of HIV-1 infection of gut or brain cells, other than those of hematopoietic origin, in vivo (5, 25, 33, 36). However, it is not yet evident whether infection of cell lines in vitro is relevant to tissue-specific pathogenesis, such as enteropathy and dementia, in AIDS. The experiments reported here and previously (9) have been restricted to the interaction of established laboratory strains of HIV-2 with immortalized CD4-negative cell lines. The results presented by Harouse et al. (19, 20) suggest that galactocerebroside could serve as a receptor for the infection of oligodendrocytes, which were noted to be infected in one study of brain biopsy specimens (47). The putative CD4-independent glycoprotein receptor described here may play a related or additional role in HIV-2 entry into CD4 negative cells in vivo. Further investigations involving HIV-2 infection of primary-cell cultures and tissue tropism, comparing LAV-2_{ROD} with LAV-2/B, could be considered.

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